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ROLE OF COX-2 IN THE BIOACTIVATION OF METHYLENEDIANILINE AND IN ITS PROLIFERATIVE EFFECTS IN VASCULAR SMOOTH MUSCLE CELLS

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Abstract

4,4'-Methylenedianiline (DAPM) is an aromatic diamine used directly in the production of polyurethane forms and epoxy resins, or as a precursor to MDI in the manufacture of some polyurethanes. In our prior experiments, we showed that chronic, intermittent treatment of female rats with DAPM resulted in vascular medial hyperplasia of pulmonary arteries. In addition, treatment of vascular smooth muscle cells (VSMC) in culture with DAPM increased rates of proliferation in a manner that was inhibited by cotreatment with *N*-acetylcysteine but was not associated with oxidative stress. We thus hypothesized that NAC treatment inhibited DAPM toxicity by competing for binding reactive intermediates formed through DAPM metabolism. Because the peroxidase enzyme cyclooxygenase is constitutively expressed in VSMC, and because cyclooxygenase is known to metabolize similar aromatic amines to electrophilic intermediates, we further hypothesized that DAPM-induced VSMC proliferation was dependent upon COX-1/2-mediated bioactivation. To test this hypothesis, we treated VSMC with DAPM and measured cell proliferation, COX-2 expression, COX-1/2 activity and levels of covalent binding. DAPM treatment resulted in a dose-dependent increase in proliferation that was abolished by cotreatment with the COX-2-selective inhibitor celecoxib. In addition, DAPM exposure increased rates of proliferation in VSMC isolated from wildtype but not COX-2 (–/–) mice. Paradoxically, treatment with DAPM reduced cellular production of PGE₂ and PGF_{2α}, but dose-dependently increased COX-2 protein levels. Covalent binding of [¹⁴C]-DAPM to VSMC biomolecules was greater in wildtype than in COX-2 (–/–) cells. However, covalent binding of [¹⁴C]-DAPM was not altered by cotreatment with a nonselective inhibitor of cytochromes P450. These studies thus suggest that DAPM-induced VSMC proliferation may be due to bioactivation of DAPM, perhaps through the action of cyclooxygenase. The data furthermore suggest that DAPM's mechanism of action may possibly involve inhibition or suicide inactivation of COX-2. In addition, because we observed an increase in DAPM-induced VSMC proliferation in cells isolated from female compared to male rats, further studies into the potential interplay between DAPM, the estrogen receptor, and COX-2 seem warranted.

Keywords

vascular smooth muscle cell proliferation; aromatic amine; bioactivation; cyclooxygenase; peroxidase; metabolism

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INTRODUCTION

4,4'-Methylenedianiline (DAPM) is an industrial compound used either directly in the production of polyurethane foams for insulating material, coating materials, Spandex fibers, dyes and epoxy resins, or as a precursor to 4,4'-methylenediphenyldiisocyanate (MDI) for the production of a large number of other polyurethane products (1). OSHA estimates that approximately 600 million pounds of MDA are produced for synthesis of MDI. However, an estimated 4,474,000 pounds are produced in the U.S. for sale, and ~ 1.8 million pounds are imported from elsewhere (2).

While the hepatobiliary toxicity of methylenedianiline is well-known (3–5), in our prior studies, we demonstrated that once weekly treatment of female rats for 16–20 weeks produced medial hyperplasia of pulmonary arteries (6), suggesting a DAPM-induced proliferation of vascular smooth muscle cells (VSMC). The observed pathology was reminiscent of the human pathological condition pulmonary hypertension. VSMC proliferation could also be induced by *in vitro* treatment with DAPM (6), suggesting that hepatic metabolism may not be required for DAPM-induced VSMC proliferation. Treatment with N-acetylcysteine (NAC), an antioxidant thiol, attenuated VSMC proliferation, but its protective effects did not involve a reduction in cellular oxidative stress, since DAPM did not itself produce an oxidant injury (6). Thus, we hypothesized that the protective effects of the NAC may be due to detoxication of reactive DAPM metabolites, e.g. electrophiles, produced in VSMC. VSMC express both P450's as well as peroxidase enzymes, namely, cyclooxygenases 1 and 2. Both COX-1 and COX-2 are constitutively expressed in VSMC, and as peroxidase enzymes, are known to participate in the metabolism and bioactivation of aromatic amines like DAPM (7). Thus, in these studies, our aim was to test whether DAPM-induced VSMC proliferation was dependent upon COX-1/2-mediated bioactivation.

METHODS AND MATERIALS

Culture and treatment of vascular cells

Primary cultures of VSMC were isolated as described previously (6) from four male Sprague-Dawley rats (~6 wks old). Cells from passages 2–7 were used for experiments. Just prior to an experiment, VSMC were trypsinized, were diluted in DMEM containing 10% fetal bovine serum (FBS), and were plated into 96-well plates. When the cells reached ~80% confluence, cell cycle was synchronized by incubating the cells with DMEM containing 0.1% FBS for 72 h. After synchronization, the cells were incubated with DMEM containing 10% FBS and either 25–100 μ M DAPM or vehicle (0.05% ethanol). The VSMC were incubated at 37°C for 24, 48, and 72 h and were assayed for VSMC proliferation by measuring rates of DNA synthesis using the BrDU (5-bromo-2'-deoxyuridine) Labeling and Detection Kit I (Roche Molecular Biochemicals, Indianapolis, IN).

In some experiments, the cells were co-treated with 2 nM of the COX-2-selective inhibitor celecoxib. The dose of celecoxib utilized for these co-incubation experiments was carefully selected. Our intent was to utilize a small dose of celecoxib, such that the celecoxib itself did not induce effects on VSMC proliferation independent of DAPM treatment. Because literature reports for the IC₅₀ for celecoxib vary depending upon cell type, we began by incubating VSMC with increasing doses of celecoxib over 24h. The levels of PGE₂ in the medium were then determined using an ELISA kit from Cayman Chemicals (Ann Arbor, Michigan). The resulting data indicated that in our cell system, the IC₅₀ for celecoxib was 30 nM. Moreover, we found that a 2 nM dose itself exhibited no effect on VSMC proliferation. Thus, the 2 nM dose was selected for our co-incubation experiments.

To further test whether COX-2 protein was necessary for the vascular proliferative responses induced by DAPM treatment, we isolated aortic VSMC from 6 male COX-2 knockout mice (B6;129P2-Ptsg2^{tm1Smi}; Taconic) compared to 6 of their wildtype littermates (also male). We treated each simultaneously in culture with vehicle or DAPM for 0–72h and then measured rates of DNA synthesis using the BrdU incorporation assay. The resulting data were expressed as a percent of that observed for vehicle treatment of cells from the same cell type (i.e., COX-2-deficient compared to wildtype).

Finally, to test whether DAPM-induced effects observed in cells isolated from male rats were maintained or exacerbated in cells isolated from females, in some experiments, VSMC were isolated from the aortas of 6 female Sprague Dawley rats (~6 weeks of age). For these studies, we utilized the same isolation protocol we reported earlier for male rats (6), except that in this case, the duration of the elastase/collagenase digestion procedure was not longer than 30 min. All other cell culture and DAPM treatment paradigms were precisely as described for VSMC isolated from male rats.

Determining levels of prostaglandin production

Levels of TXB₂ (the stable metabolite of TXA₂), PGE₂ and PGF_{2α} were measured in the medium of DAPM-exposed cells, using ELISA kits purchased from Cayman Chemical (Ann Arbor, Michigan). These levels were normalized to the level of cell protein in each well, with protein concentrations measured using the BCA assay (Thermo Scientific, Rockford, IL). Levels were measured at the 24h time point, because we needed to allow for accumulation of sufficient levels of prostaglandin in the medium so as to be able to differentiate effects between treatment groups. This time point also seemed biologically relevant, as this was the same time point where significant decreases in VSMC proliferation were detected.

Western blot analysis for COX-2 protein levels

To determine whether DAPM treatment culminated in an increase in COX-2 protein, we treated VSMC with DMEM growth medium containing 0–25 μM DAPM for 0–24 h and measured COX-2 protein levels using western blot analysis. The VSMC were lysed by incubation in 50 mM Tris-HCl, containing 300 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail. The cells were removed from the plates by scraping, and the protein concentration in each sample was determined using the BCA protein assay (Thermo Scientific, Rockford, IL). The cell lysates were diluted in Laemmli buffer and were incubated 5 min at 100°C, and 5 μg sample or COX-2 standard (Cayman Chemical) was applied to each lane of a Novex 4–20% Tris-glycine gradient gel (Invitrogen, Carlsbad, CA). The gel was electrophoresed for 2h at 125V and then transferred onto a PVDF membrane for 1h at 100V. The membrane was blocked overnight at 4°C, and after washing twice, was incubated for 2h with a 1:500 dilution of COX-2 polyclonal antibody (rabbit α mouse; Cayman Chemical) or ERK 1/2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/Tween buffer. After rinsing twice more, the membrane was incubated with a 1:5000 dilution of secondary antibody (α rabbit, for COX-2, or α mouse, for ERK 1/2; GE Healthcare) for 1 h, and after two additional washes, was developed using the ECL plus detection system (GE Healthcare). The blots were developed on X-ray film. The film was imaged, and densitometric analysis was performed using a BioRad VersaDoc system (Hercules, CA). To confirm that equivalent amounts of protein were loaded into each well, the bands were normalized to total ERK protein.

Assessing DAPM metabolism in VSMC

To determine whether VSMC COX-2 was responsible for DAPM metabolism in VSMC, we treated COX-2-deficient compared to wildtype VSMC with 4 μM [¹⁴C]-DAPM (American

Radiolabeled Chemicals, St. Louis, MO; specific activity = 14 mCi/mmol) and then separated DAPM metabolites using a Waters (Milford, MA) 626 HPLC interfaced to an INUS Systems (Tampa, FL) β -RAM radioisotope detector. The chromatography was accomplished using a 25 cm Beckman Ultrasphere reversed phase C18 column, a flow rate of 0.5 mL/min, and a gradient from 80% 20 mM ammonium acetate, pH 5.0/20% acetonitrile to 50% ammonium acetate/50% acetonitrile over 40 minutes.

Measuring covalent binding to DNA and protein in VSMC

COX-2 deficient compared to wildtype VSMC were treated 4 μ M with [14 C]-DAPM and covalent binding to cell biomolecules (i.e., protein, DNA, and RNA) was determined as we described previously (5). In some experiments, VSMC were co-treated with 100 μ M SKF525A (Proadifen; Sigma Chemicals, St. Louis, MO), a general P450 inhibitor.

Statistics

Data are expressed as means $\pm$ SEM and were analyzed by two-way analysis of the variance using SPSS version 10.0 (SPSS Inc., Chicago, IL) or GraphPad Prism 5.0 (La Jolla, CA) software. When statistical significance was detected (i.e., $p < 0.05$), Tukey's posthoc t-tests were conducted to determine differences between individual data points.

RESULTS

Effects of COX-2 on DAPM-induced VSMC proliferation

In VSMC treated with 5–50 μ M DAPM, rates of proliferation, assessed as increases in DNA synthesis, were dose-dependently increased by 8–25% at 24–72h after treatment (Figure 1). In our prior experiments, similar increases in BrDU incorporation in DAPM-treated cells resulted in ~20% increases in cell number (6). In experiments aimed at testing whether COX-2 had a causal role in DAPM-mediated VSMC proliferation, we treated cells with 25 or 50 μ M DAPM $\pm$ the COX-2 selective inhibitor celecoxib. In these experiments, treatment with 25 μ M DAPM increased BrDU incorporation in VSMC by 15–30% at 24–72 h after treatment (Figure 2). Rates of DNA synthesis were not significantly affected by treatment with celecoxib alone, but the increased rates of DNA synthesis induced by DAPM were abolished by the celecoxib co-treatment (Figure 2). Similar results were observed for the 50 μ M dose (not shown).

In the interest of utilizing multiple approaches for assessing the role of COX-2 in DAPM-mediated VSMC proliferation, VSMC isolated from COX-2 (–/–) mice compared to their wildtype littermates were treated with 25 or 50 μ M DAPM and rates of DNA synthesis were again measured. In this case, DAPM treatment induced a 20–38% increases in cell proliferation over 72h; however, these DAPM-induced effects were completely absent in the COX-2-deficient cells (Figure 3).

Effects of DAPM on prostaglandin production and COX-2 expression

To first determine whether DAPM treatment altered COX-2 protein levels, we treated VSMC with DMEM growth medium containing 0–25 μ M DAPM for 0–24h and measured COX-2 protein using Western blot analysis. The densitometric data indicated that COX-2 protein was increased by 50–100% within 4–8h after DAPM treatment (compare Figs. 4B and C), but the COX-2 protein levels returned to baseline by 12 h.

To test whether effects of DAPM were mediated by COX-2-dependent prostaglandin production, VSMC isolated from wildtype compared to COX-2(–/–) mice were treated with 25 or 50 μ M DAPM, and TXA₂, PGE₂ and PGF_{2 α} were measured in the spent medium at 24h. First, baseline levels of PGE₂ and PGF_{2 α} released into the medium of COX-2-

deficient cells were approximately ~70 and 55% less than that of the wildtype cells, respectively, while levels of TXB₂ were not significantly different. Treatment with 25 μ M DAPM reduced the levels of PGE₂ and PGF_{2 α} by 75% in the wildtype cells (Figure 5) but exhibited no effect on these levels in the COX-2-deficient cells. DAPM treatment also had no effect on the levels of TXB₂, in either the wildtype or the COX-2-deficient cells.

Bioactivation of DAPM

To next determine whether VSMC COX-2 was responsible for DAPM metabolism in VSMC, we treated COX-2 deficient compared to wildtype VSMC with 3.57 μ M [¹⁴C]-DAPM and separated DAPM metabolites using HPLC with radioisotope detection. We observed 3 putative DAPM metabolites in these cells. In our prior studies (8), we had identified one as *N*-acetyl DAPM and one minor metabolite as *N,N'*-diacetyl DAPM. On the assumption that a proximate toxicant of DAPM may be produced at levels below the detection limit or may be too rapidly conjugated to protein or other biomolecules so as to be difficult to detect, we compared the disappearance of DAPM in COX-2 deficient to that observed in wildtype cells (Figure 6A). However, although DAPM levels were reduced by ~50% in both cell types over 24h, no difference in the rate of disappearance of DAPM was detected between the COX-2(–/–) and wildtype cells.

To next determine whether DAPM was bioactivated in VSMC so as to induce its binding to DNA and protein, covalent binding of [¹⁴C]-DAPM was determined. As shown in Figure 6B, covalent binding in COX-2-deficient VSMC was only 40% of that measured in wildtype cells. However, treatment with SKF525A produced no significant change in the covalent binding of [¹⁴C]-DAPM.

DAPM-induced effects on cell proliferation in VSMC isolated from male compared to female rats

To assess whether DAPM-induced effects were maintained or even exacerbated in VSMC isolated from female rats compared to those isolated from males, we treated female VSMC precisely as described for males, and then measured rates of DNA synthesis using the BrdU incorporation assay. Results indicated that DAPM-induced effects in the female cells were similar to that observed in VSMC isolated from males, except that the effects were somewhat greater (Figure 7). ANOVA revealed a significant effect of treatment, sex and time. Note that for clarity of the presentation, only the 50 μ M dose of DAPM was shown, but similar sex-dependent differences were observed for DAPM doses of 10 and 25 μ M.

DISCUSSION

A wealth of literature indicates that cyclooxygenase-derived prostaglandins regulate VSMC proliferation. Two isoforms of COX exist – COX-1 and the inducible form, COX-2. Though COX-2 is constitutively expressed in VSMC (9), various stimuli (e.g., proinflammatory cytokines) can increase the expression of COX-2 (10), promoting greater levels of prostaglandin synthesis. Evidence linking cyclooxygenase expression and/or activity with vascular diseases include its pronounced expression in atherosclerotic lesions (11) and the induction of COX-2 following balloon angioplasty (12). In addition, continuous intravenous infusion of prostacyclin is a well-recognized therapy for pulmonary arterial hypertension (13). Prostaglandins have been shown to influence smooth muscle cell growth/proliferation, perhaps through modulation of the transition from the G₀/G₁ to the S phase of the cell cycle (14). The effects of the various prostaglandins on proliferation, however, are varied. For example, prostaglandin E₂ (PGE₂) can either inhibit (15) or stimulate (16) proliferation, PGI₂ (mainly produced by endothelial cells) inhibits proliferation (17), while thromboxane

A₂ stimulates proliferation (18). The COX-2 pathway is argued by some to be a negative feedback mechanism, protecting the vasculature against VSMC proliferation (19). In support of this view, COX-2 inhibition has been shown to promote pulmonary vascular remodeling and contractility in the hypoxia-induced hypertension model (20). Other work has suggested that COX-2 upregulation stimulates VSMC proliferation (9). In support of these findings, COX-2 inhibition has been shown to reduce IL- β -mediated VSMC proliferation (21). To complicate the issue further, clinical findings correlating the use of the COX-2-selective inhibitor rofecoxib with an increased risk of cardiovascular disease (22) certainly suggest that COX-2 is vascular-protective. However, the presumed vascular protective effects of COX-2 in this case are likely mediated by its role in producing platelet-derived prostacyclin (23). While the role of COX-2 in regulating VSMC proliferation appears complex, the role of COX-1 in modulating vascular protection seems more straightforward. The clinical efficacy of aspirin, a nonselective COX inhibitor, in decreasing the risk of atherosclerosis (24, 25) is well-appreciated. Furthermore, its predominate protective mechanism appears to be its ability to inhibit COX-1-derived thromboxane production in platelets (23). In addition, treatment with a selective COX-1 inhibitor (SC-560) was shown to decrease intimal hyperplasia in rats following balloon angioplasty (12), and in LDL-receptor knockout mice, COX-1 was shown to have a role in atherosclerotic plaque formation (26).

Regardless of whether COX-2 expression itself promotes or inhibits VSMC mitogenesis, in these studies, COX-2 clearly had a role in promoting DAPM-induced VSMC proliferation. Specifically, we showed that DAPM induces VSMC proliferation in a dose-dependent manner, and that this proliferation is blocked in the absence of COX-2 activity or expression. We used two approaches for determining these COX-2-dependent effects. We first utilized co-incubations with a very low dose of a pharmacological inhibitor of COX-2, and these experiments clearly indicated that DAPM-induced VSMC proliferation was abolished by co-treatment with a COX-2 inhibitor (Figure 2). To confirm these results, we also determined DAPM-induced effects in cells isolated from wildtype compared to COX-2-deficient mice. In these experiments, DAPM-induced proliferation was completely blocked in the absence of COX-2 (Figure 3). Thus, through two different methods, we showed that DAPM-induced proliferation was blocked in the absence of COX-2 activity/expression. One caveat of these studies is that we measured DAPM-induced effects relative to the effect of vehicle in each cell type (i.e., wildtype compared to COX-2 deficient cells). Though we did not directly compare the growth of wildtype VSMC with that of COX-2 deficient cells, we did note that during culture, the COX-2-deficient cells tended to grow a bit more slowly than cells isolated from wildtype animals. This suggests that COX-2 expression/activity can positively regulate VSMC proliferation. This is in agreement with prior studies that also demonstrated a role for COX-2 in VSMC proliferation (21).

We initially had two potential hypotheses for the dependence of DAPM toxicity on COX-2 protein expression. First, COX-2 is an integral component of a number of signal transduction pathways that modulate cell proliferation. Thus, COX-2 induction may serve to directly modulate signaling and by extension, cell proliferation. On the other hand, COX-2 is a peroxidase enzyme; thus, it can utilize aromatic amines as substrates in the co-oxidation reactions involved in its synthesis of PGH₂. Specifically, in these reactions, the hyperperoxide PGG₂ is reduced to form PGH₂. In the process, the aromatic amine is oxidized, typically through two sequential 1-electron oxidations (7). Thus, peroxidase enzymes such as COX-1/2 typically generate electrophilic metabolites and often form a free radical species as an intermediate (7). Since DAPM is an aromatic amine, and because both COX-1 and COX-2 are constitutively expressed in vascular smooth muscle, we hypothesized that DAPM metabolism through COX-1/2 may also be important, leading to the production of electrophilic intermediates that bind to cellular protein or DNA. In support of this hypothesis, evidence of DAPM metabolism by peroxidase enzymes has been

demonstrated previously (27). In addition, COX-2 has been shown to be involved in the metabolism and bioactivation of benzidine, a molecule structurally very similar to DAPM (28). Moreover, in our earlier studies, DAPM-induced proliferation was inhibited by co-treatment with *N*-acetylcysteine (NAC), but this effect was not related to NAC's ability to modulate cellular oxidative stress levels (6). Thus, we rationalized that perhaps NAC prevented DAPM-induced effects potentially through its binding to reactive intermediates formed through DAPM metabolism in VSMC.

To test whether VSMC COX-2 had a role in DAPM metabolism in VSMC, we treated COX-2 deficient compared to wildtype VSMC with [¹⁴C]-DAPM and separated DAPM metabolites using HPLC with radioisotope detection. Though we did observe DAPM metabolites in these cells, they were the same acetylated metabolites as we had previously reported - *N*-acetyl DAPM and one minor metabolite as *N,N'*-diacetyl DAPM (8). While we expect that neither of these metabolites were responsible for DAPM-induced toxic effects, to try and unveil whether other metabolites not yet detected might also be formed, we compared the disappearance of DAPM in COX-2 deficient as compared to wildtype cells. However, no difference was observed, indicating that DAPM was not spared when COX-2 was absent. It is nevertheless still possible that another metabolite produced by COX-2 might indeed exist in the VSMC, but 1) it binds to protein so readily that it is not detectable unbound or in free form in homogenates, and 2) it is formed at levels far below that of the parent compound, thus having little impact on the rate of disappearance of DAPM. In support of this view, our covalent binding studies indicate that only ~4% of the DAPM applied to the cells was bound to cell protein and DNA. However, in COX-2-deficient cells, covalent binding of [¹⁴C]-labeled DAPM was only 40% of that measured in wildtype cells, while treatment with a nonselective P450 inhibitor did not alter covalent binding. Taken together, these data suggest the possibility that COX-2 may possibly be involved in the bioactivation of DAPM in VSMC.

Also of note was that although DAPM increased COX-2 expression 6–8h after the initiation of treatment, prostaglandin production, particularly that of PGE₂ and PGF_{2α}, was significantly decreased. Given our data suggesting COX-2 dependent effects on covalent binding of DAPM to VSMC protein and DNA, we speculate that this apparent inhibition in prostaglandin production may possibly be due to a suicide inactivation of COX-2. Although neither COX-1 nor COX-2 directly produces these prostaglandins, their expression/activity is directly linked to their subsequent synthesis by the prostaglandin synthases. In addition, the synthesis of particular prostaglandins is generally coupled to a given COX isoform, often due to the cellular co-localization of the COX isoform with a given prostaglandin synthase. For example, in platelets, COX-1 is coupled to TXA₂ production, while COX-2 is intimately linked with PGI₂ synthesis (23). COX-2 in VSMC has been shown coupled to PGE₂ production (29). On the other hand, others have shown that the cytosolic PGE synthase (cPGES) utilizes PGH₂ synthesized by COX-1, while the membrane-associated PGES-1 (mPGES-1) uses PGH₂ produced by COX-1 (30). Thus, the coupling of COX-1 or COX-2 to the synthesis of a given prostaglandin may be case or cell-type specific. Nevertheless, in these studies, TXA₂ production seems more closely linked with COX-1, since baseline levels of its stable metabolite, TXB₂, were not reduced in COX-2-deficient cells (Figure 5C). On the other hand, COX-2 expression appears coupled at least in part with the synthesis of PGF_{2α} and PGE₂, as their baseline levels were diminished by ~55 and 70%, respectively, in COX-2-deficient compared to wildtype cells (Figures 5A and B). Furthermore, DAPM treatment selectively inhibited the production of these two prostaglandins (Figures 5A and B), even though COX-2 expression was increased at 6–8h (Figure 4). It is possible that DAPM-induced increases in VSMC proliferation may be at least in part due to its combined effect on the levels of the various prostaglandins produced within the cells.

An additional caveat of these studies is that, for convenience, we utilized cells isolated from male animals. However, our data suggest that DAPM elicits similar effects in cells isolated from female rats, although the effects are somewhat more exaggerated in cells isolated from females (Figure 7). Moreover, our prior *in vivo* studies suggested that with a more chronic treatment, similar vascular proliferative effects can eventually be observed in male animals (6). However, it is possible that estradiol and estrogen receptor (ER) activation interact to exacerbate the vascular toxic responses of DAPM. COX activity and expression are modulated by estradiol, with estradiol increasing cyclooxygenase-dependent arachidonic acid metabolism (31, 32). Although estradiol can increase the expression of COX-2 (33), one report suggests that estradiol can also increase the expression of COX-1 (34). In addition, the estradiol-dependent modulation of vascular COX-2 expression is dependent upon binding to estrogen receptor- (ER (35). Thus, if DAPM selectively targets the COX-2 pathway in VSMC, the presence of estradiol may further exacerbate its vascular toxic effects. Thus, while we have demonstrated a role for cyclooxygenases in DAPM-induced VSMC proliferation, and have shown in earlier studies that the vascular proliferative responses to DAPM exposure are observed more prominently and at an earlier time point in pulmonary arteries of female rats, a detailed study examining the interaction between COX-2 and the ER in DAPM-mediated VSMC appear warranted.

In summary, while our earlier studies demonstrated that chronic, oral DAPM treatment can promote vascular medial hyperplasia in pulmonary arteries of exposed rats, these and our prior studies have shown that *in vitro* treatment of VSMC with DAPM can induce mitogenesis. In these studies, we've shown that DAPM-induced VSMC proliferation is COX-2 dependent, and that COX-2 may possibly have a role in the bioactivation of DAPM in these cells. Finally, DAPM-induced VSMC proliferation may be related to its inhibition of COX-2 activity and the concomitant decreases in levels of certain prostaglandins. Importantly, a better understanding of DAPM-induced pulmonary vascular toxicity in female animals may reveal new insights into the onset of pulmonary hypertension in females. Given that adult females tend to have a higher risk for developing pulmonary hypertension compared to adult males, such studies could perhaps suggest new molecular targets for therapy.

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Figure 1. Effects of 4,4'-methylenedianiline (DAPM) on vascular smooth muscle cell proliferation. Proliferation of cells was assessed as rates of DNA synthesis, determined by measuring incorporation of BrDU. Data are means $\pm$ SEM and are expressed as a percent of BrDU incorporation measured for cells treated with vehicle. Two-way ANOVA revealed a significant effect of time and treatment. *Denotes that all points are significant compared to vehicle at the same time point.

Figure 2.

Effects of co-treatment with a COX-2 selective inhibitor on DAPM-induced VSMC proliferation. VSMC were treated with 25 μ M DAPM or DAPM + 2 nM celecoxib, and DNA synthesis was assessed using the BrDU incorporation assay. Data are expressed as a percent of that measured for treatment with vehicle only, and represent means $\pm$ SEM for n=3 experiments. Two-way ANOVA revealed a significant effect of treatment and time. *Denotes significance compared to treatment with vehicle only. Note that similar results were obtained for treatment with 50 μ M DAPM $\pm$ 2 nM celecoxib (not shown).

Figure 3.

Rates of DNA synthesis determined in VSMC isolated from wildtype compared to COX-2- $(-/-)$ mice treated $\pm$ 25 μ M DAPM. Cell proliferation was assessed as rates of DNA synthesis, determined using the BrDU incorporation assay. Data represent means $\pm$ SEM for $n=3$ experiments. Two-way ANOVA revealed a significant effect of treatment with DAPM and significant differences between wildtype and COX-2 deficient cells. *Denotes significant increases compared to treatment with vehicle for the same time point. Note that similar results were obtained for treatment with 50 μ M DAPM (not shown).

Figure 4.

DAPM-induced effects on COX-2 protein levels in VSMC. A) VSMC were treated with 0–25 μ M DAPM for 8h, and COX-2 compared to total ERK protein levels were measured by Western blot analysis. B) Densitometric analysis of digitized images of the blots was used to quantitate changes in COX-2 protein after treatment with DAPM. These changes were normalized to that observed for total ERK and were expressed as a percent of that observed from treatment with vehicle. C) Densitometric analysis of blots comparing effects of 5 and 10 μ M DAPM at 0–24h after treatment. For B) and C), data represent means $\pm$ SEM for n=5 experiments. Two-way ANOVA revealed significant effects of dose and time. *Denotes significant increases in COX-2 protein compared to either B) treatment with vehicle only, or C) the 0h timepoint.

Figure 5.

DAPM-mediated effects on prostaglandin production in wildtype compared to COX(-/-) VSMC. VSMC were treated with 25 μ M DAPM and PGE₂ (top), PGF_{2α} (middle) or TXB₂ (bottom) levels were measured by enzyme linked immunoassay of spent medium collected at 24h. Data represent means $\pm$ SEM for n=3 experiments. Two-way ANOVA revealed a significant effect of treatment and strain for PGE₂ and PGF_{2α}. *Denotes significance compared to wildtype cells treated with vehicle.

Figure 6.

Effects of cyclooxygenase on DAPM metabolism and covalent binding. A) Wildtype or COX-deficient VSMC were treated with 3.6 μ M [¹⁴C]-DAPM for 0–24 h. Concentration of DAPM was measured in homogenates of cells plus medium using HPLC with radioisotope detection. Data are expressed as means $\pm$ SEM for n=3 experiments. Two-way ANOVA indicated no significant effect of strain or time. B) Covalent binding of [¹⁴C]-DAPM was measured in wildtype or COX-deficient VSMC treated with 3.6 μ M [¹⁴C]-DAPM for 0–24 h. In some experiments, cells were co-treated with 100 μ M SKF525A, to inhibit cytochrome P450. Data represent means $\pm$ SEM for n=3 experiments. Two-way ANOVA with Tukey's posthoc test revealed a significant effect of strain. *Indicates p <0.05, compared to wildtype cells treated with vehicle.

Figure 7.

Effects of DAPM on cell proliferation in VSMC isolated from male compared to female rats. VSMC isolated from aortas of male and female rats were synchronized by serum deprivation and then were stimulated by incubation in DMEM containing 10% FBS and 0–50 μ M DAPM. Cell proliferation was assessed at 24, 48 and 72h by determining levels of DNA synthesis using the BrdU incorporation assay. For clarity, only the 50 μ M dose of DAPM is shown, but doses of 10 and 25 μ M DAPM showed similar results. ANOVA with Tukey's posthoc tests revealed a significant effect of time, treatment and sex. Rates of DNA synthesis were increased compared to vehicle at all time points in both male and female VSMC. *Indicates significant differences ($p < 0.05$) between male and female VSMC for a given time point.